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PRINCIPAL INVESTIGATOR: William G. Cance, M.D.

CONTRACTING ORGANIZATION: North Carolina University at Chapel Hill
Chapel Hill, North Carolina 27599-4100

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13. ABSTRACT *(Maximum 200 words)*

This project focuses on the biology of the Rak protein tyrosine kinase in human breast cancer. Rak is a novel nuclear tyrosine that our group has identified in breast cancer tissues and cell lines that has structural homology to the Src tyrosine kinase, with SH2 and SH3 domains at its amino terminus. Rak appears to be a potent growth inhibitory gene in breast cancer, as in induction of expression in Rak breast cancer cells led to detachment from their substratum, and subsequent apoptosis as determined by TUNEL assays. We are currently developing an adenoviral system of Rak gene delivery to human breast cancer cells to further characterize this effect. During the past year, we have developed monoclonal antibodies against Rak which recognize the 54kDa Rak protein in breast cancer cells. Preliminary studies indicate that Rak is expressed in normal breast epithelium, and in a subset of human tumors. Further characterization of the expression of Rak in larger numbers of human breast tissues will be conducted to understand the biology of this kinase in breast cancer and determine its suitability as a therapeutic target.

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William K. Rose MD 7/27/98

PI - Signature Date

TABLE OF CONTENTS

I. INTRODUCTION

This research grant focuses on the biology of protein kinases in human breast cancer. It appears that several protein tyrosine kinases are implicated in the progression of human breast cancer, including HER2/*neu* and EGFR. We have isolated novel protein kinases from human breast cancer cells using low stringency PCR amplification of the consensus sequences contained within their kinase domains (*1,2*). The current work has focused on the Rak tyrosine kinase. Rak is a novel nuclear tyrosine kinase identified in breast cancer tissues and cell lines that has structural homology to the Src tyrosine kinase, with SH2 and SH3 domains at its amino terminus(*3*). In addition, Rak has been shown to bind to the Rb gene product(*4*). The Src family of tyrosine kinases localizes primarily to the cell membrane, where they are thought to play a role in mitogenic signaling. The sequence of Rak revealed that it does not have an amino terminal region which targets its localization to the cell membrane. Rather, Rak has a putative nuclear localization sequence within its SH2 domain, and we have shown that Rak localizes to the nucleus. We suspected that the novel localization of Rak suggested that Rak might have a different role from other Src-related kinases, and we subsequently found that Rak has growth inhibitory activity in a number of cell lines.

We were particularly excited by work presented in our Annual Report from last year, which was also presented at the Era of Hope meeting last year in November. In these experiments, Rak was transfected into a panel of breast cancer cell lines. Our rationale was that these cell lines express low levels of Rak, and perhaps they contain some mechanism that prevents Rak from being toxic. If we could express Rak in these cells, then we could gain a better understanding of Rak's localization, regulation, and role in cell signaling. However, when we attempted to transfect Rak into breast cancer cells, they lost adhesion, rounded up, and floated from the dish within 24 hours. This is a greater degree of sensitivity to Rak than was exhibited by any other set of cell lines that we have studied. We feel that these results suggest a potential for Rak as a therapeutic target in breast cancer cells, and we described last year our plans to prepare a system in which Rak expression could be induced in breast cancer cells. Those experiments are described in the next section under Specific Aim 1.

We have found that Rak expression leads to apoptosis following induction in the BT474 breast cancer cell line. However, our work was frustrated by a strong counterselection against cells capable of expressing Rak. Specifically, when we first isolated cells containing an inducible Rak construct, Rak could be expressed and we observed this strong phenotype. However, when the cells were continually passaged, even for a short amount of time, we lost the ability to induce Rak expression. For this reason, we have turned to a new approach to introduce Rak into breast cancer cells. Rak has been cloned into an adenoviral construct, and we are preparing an adenovirus that will efficiently deliver Rak into cells. That work is described in the progress on Specific Aim 1.

As a second Specific Aim, we have attempted to analyze the expression of Rak in breast tumors. We have previously found that in a small set of samples (9 tumors), Rak expression was decreased relative to its expression in normal tissue. In addition, we have found that localizes to chromosome 6q21, a region that is frequently deleted in breast and

ovarian cancers. To examine Rak expression more thoroughly, we have prepared monoclonal antibodies to Rak. This work is described in detail in the next section under Specific Aim 2. Our plan is to use these antibodies to determine the expression and localization of Rak in a panel of cell lines and tumors. Early results indicate that Rak expression is indeed decreased in tumors relative to normal tissue, and these results will also be discussed below.

Finally, we plan to pursue the significance of Rak's chromosomal localization later in the time frame of this project, and will discuss our plans below.

II. BODY

Technical Objective 1. Isolate and characterize breast cancer cell lines containing the Rak kinase under control of an inducible promoter

A. Development of inducible systems of Rak expression.

We have attempted to express Rak in a variety of cell lines, and have found that Rak has a toxic phenotype that is detected as an initial arrest in the G2/M phase of the cell cycle, and a subsequent loss of viability to Rak expressing cells. Following this loss of viability, Rak-expressing cells could no longer be detected in the population. For this reason, we subsequently attempted to place Rak under the control of a dexamethasone-inducible promoter, but this resulted in a "leaky" regulation of expression, and following a limited period of sub-culturing, Rak-expressing cells appeared to be counterselected from the population.

For this reason, we have turned to the metallothionein promoter, which is inducible by the addition of zinc sulfate to the medium in which the cells are cultured. Fortunately, our lab has developed extensive experience in inducing the expression of another gene, the carboxy-terminal domain of the focal adhesion kinase. Although this domain, which we call FAK-CD, is toxic when constitutively expressed under the cytomegalovirus promoter present in a number of expression plasmids, we have been able to induce the expression of FAK-CD in BT474 breast cancer cells and observe the resultant phenotype. This work has recently been submitted for publication, and is funded under a separate grant through the NIH.

We wished to place Rak under the control of the metallothionein promoter, induce its expression in BT474 cells, and determine if Rak expression was toxic to breast cancer cells. A version of Rak, which included a 5' FLAG sequence, was cloned into the pMEP4 plasmid (InVitrogen) from pBK-CMV (Stratagene) using 5' NheI and 3' XhoI sites. pMEP4 encodes hygromycin resistance and contains a zinc-inducible metallothionein promoter to drive foreign gene expression. The FLAG sequence encodes an epitope tag to distinguish the exogenously expressed Rak from the Rak which is weakly expressed in the BT474 cells under normal conditions. Following induction of Rak, the protein can be detected using a commercially available monoclonal antibody to FLAG.

To generate stably transformed cell lines, pMEP4-Rak is introduced into the cells by transfection using the lipofectamine reagent (Gibco BRL) and serum free media. The cells are incubated overnight, and the next morning lipofectamine and excess DNA are washed away with PBS and replaced with complete media. The cells are incubated for several days until they are >80% confluent, then are split into complete media containing hygromycin and incubated until isolated colonies can be picked. Once these colonies are picked, they can be easily scaled up to 100 mm plates by normal cell passage but must always be kept in media containing hygromycin to select for cells which contain the inducible plasmid. Once several clones are obtained, the foreign gene expression is induced by addition of Zn_2SO_4 (the concentration of which was empirically determined on non-transfected cells prior to the induction experiment).

Eight clones were generated from isolated colonies and induced with 75μg/ml Zn_2SO_4. Harvested lysates were subsequently analyzed by Western blots, demonstrating that six of the clones expressed Rak (Figure 1). We selected clone 6 and clone 8 for further study. A time course experiment following induction revealed that each optimally expressed Rak at 8 hours after induction (Figure 2, Figure 3), and expression decreased over the next 24 hours. The critical part of the experiment was to determine the morphology of the cells that expressed Rak, and we found that cells overexpressing Rak lost adhesion and floated from the substratum. Furthermore, these cells were positive using the TUNEL assay, indicating that they had undergone apoptosis. This result was consistent with the findings we presented in our Annual Report last year- that Rak induced loss of adhesion and death in breast cancer cell lines. In addition, we wished to pursue other questions using this approach, such as the nature of Rak's growth inhibition and the proteins with which it interacts.

However, these inducible-Rak BT474 cells cannot be passaged for very long before they begin to lose their Rak expression. For example, clone BT-Rak6, which was characterized most closely, lost a significant amount of protein expression, as measured by Western analysis, between the time the clone was first confirmed and the first timecourse, a span of approximately 8 passages. Similar loss of expression was seen from BT-Rak8 after multiple passages. Repeated attempts to visualize Rak by immunofluorescence using the FLAG monoclonal and polyclonal antibodies have been unsuccessful. It is possible that this lack of expression was due to technical problems, but we have observed this same loss of Rak expression in a number of sub-clones, and we believe that Rak is strongly counterselected by tumor cells.

B. Development of adenoviral and GFP-based systems of Rak expression.

We have concluded that in order to study the effects of Rak we must move to a system whereby the gene can be efficiently introduced into the host cell and expression can be monitored without passaging the cells. The adenoviral system has worked very well for the study of another growth inhibitory gene product, FAK-CD (described briefly above), and seems like it would be a likely system to successfully study Rak because high levels of gene expression can be obtained when the breast cancer cells are infected with the adenovirus. Our strategy is to clone Rak-FLAG out of pMEP4 using the 5' KpnI and 3'XhoI site and ligate it into pACCMV-PLPASR+ (the adenoviral shuttle vector) which has been cut with KpnI and SalI (XhoI and SalI ends are compatible). We have successfully cloned RAK into this shuttle vector, and the sequence has been confirmed. We are now testing its expression in human kidney 293 cells. Once we determine we have Rak protein expressed, the shuttle vector will be co-transfected along with adenoviral DNA into the 293 cells; and a double recombination event will generate adenovirus DNA containing Rak. Recombinant adenovirus has already been shown to efficiently infect BT474 cells, so we expect this will be very good system for continuing our study of Rak expression in BT474.

As a backup adenoviral system, we are using the proprietary pAdlox adenoviral vector from the UNC Lineberger Comprehensive Cancer Center Gene Therapy Facility. We have PCR-cloned Rak from our pcDNA3 Rak construct and ligated it into the pAdlox

vector. We have confirmed the DNA sequence of Rak and are testing this construct in 293 cells. This construct will then be used to generate adenovirus by the gene-therapy facility, using their system. We are using both of these approaches to generate adenovirus, as our previous experience with this system has shown that it can be difficult to generate high-titred recombinant adenoviral preparations. Thus, we are testing both methodologies, as we need to develop a system of rapidly and reproducibly expressing Rak in human breast cancer cells to complete the studies described above.

Finally, we are testing the green florescent protein system as another method of expressing Rak in breast cancer cells and determining a phenotypic readout. We used the Rak PCR product from the pAdlox Rak cloning (described above), and cloned this into the GFP N1 vector which had been digested with SalI and KpnI. We have obtained positive clones which were sequenced, and are now being transfected into breast cancer cells. This method, if successful, will allow us to further determine the mechanism of the growth inhibitory effect of Rak induction, as it is ideally suited for rapid application of site-directed mutagenesis of the Rak construct, and the testing of the phenotype upon transfection in the breast cancer cells.

The expected result of these experiments is that Rak expression from the adenoviral vector and the GFP vector will lead to loss of viability in breast cancer cells. If this is the case, we plan to examine this activity further by analyzing the structural features of the protein responsible for this arrest. We would first like to ask if the growth arrest requires kinase activity. To this end, we have generated a mutant of Rak at position 262, making a lysine-to-arginine substitution, and have shown that this mutant lacks autocatalytic activity. We are also preparing mutants of Rak which lack the amino-terminal domains which direct protein-protein interactions, the SH2 and SH3 domains. However, we are reluctant to pursue these constructs until we have information about the activity of full-length Rak in breast cancer cells, because adenoviruses are not only time consuming to produce, but are also expensive to prepare at our Core Facility (approximately $800 per viral stock).

Technical Objective 2:
A. Prepare monoclonal antibodies for analysis of Rak in breast tumors
B. Analyze Rak expression in breast tumors by Western blot
C. Analyze Rak expression and localization in breast tumors by immunohistochemistry

As a means of monitoring Rak expression in various systems, we are also in the process of generating a series of monoclonal antibodies. Our methodology for the production of Rak monoclonal antibodies is shown in Figure 4. We have screened 768 hybridomas generated against gstRakNT, which are the amino acids 2-110 of Rak fused to the Glutathione-S-transferase gene in the plasmid pGEX-4T-1 (Pharmacia). The construction of this plasmid and purification of gstRakNT (NT=amino-terminus) were both performed according to the manufacturer's instructions, and yield large quantities of pure Rak fusion protein, which we have used as immunogen. We have identified one hybridoma which is useful in detecting Rak by Western analysis (Figure 5) and one which can be used for both immunofluorescence of BT474 cells and immunoprecipitation

from Cos7 RakNT lysates. Several other candidates were found to immunoprecipitate Rak from Cos7 RakNT lysates, but with much less efficiency.

To characterize these putative antibodies, we needed to develop a method of immunoprecipitation which yielded proteins of different molecular weights from the heavy chains of the precipitating antibody, since they stain strongly as a 54 kDa band. Unfortunately, this is exactly the molecular weight of Rak, so we cannot tell which antibodies were capable of precipitating Rak. To circumvent this problem, we have transfected simian COS-7 cells with a plasmid which drives the expression of the amino terminal 30 kDa fragment of Rak. We then lyse the cells, and test for the ability of the monoclonal antibodies to precipitate the fragment. Several of these antibodies are nearing clonality, so we should have expanded supplies of each within the next 2 months. A preliminary study of Rak expression in normal and breast cancer specimens from individual patients is shown in Figure 6. It appears that Rak is expressed, under some conditions, in breast cancers, and, it appears to be expressed in normal tissue from the same patient. We have also observed that, in some cases, Rak appears to migrate as a slightly lower molecular weight band in normal breast tissue, compared to matched breast cancer specimens. With these reagents we can study effects of Rak in a large number of breast tumors and correlate with known prognostic markers. We can also study Rak localization in tumors which are positive by Western blot. Through these studies, we hope to generate important clues as to why a growth-inhibitory gene appears to be expressed in human breast cancer, and, how we can use its growth-inhibitory functions as a breast cancer therapeutic.

Figure 1. Western blot analysis of induced RAK expression in human breast cancer cells

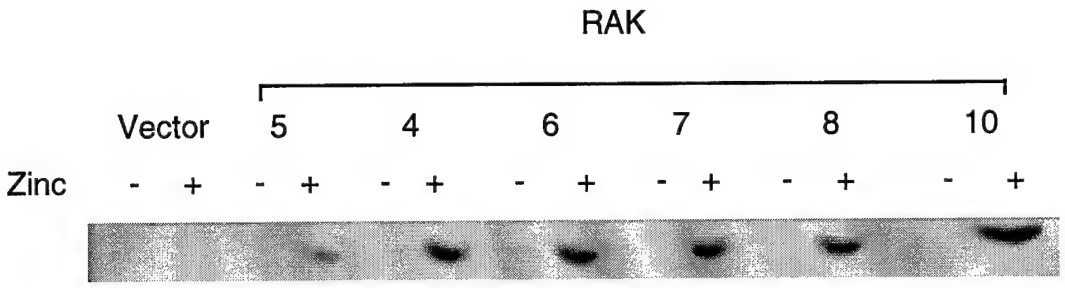


Figure 2. Western blot analysis of inducible RAK expression during a timecourse.

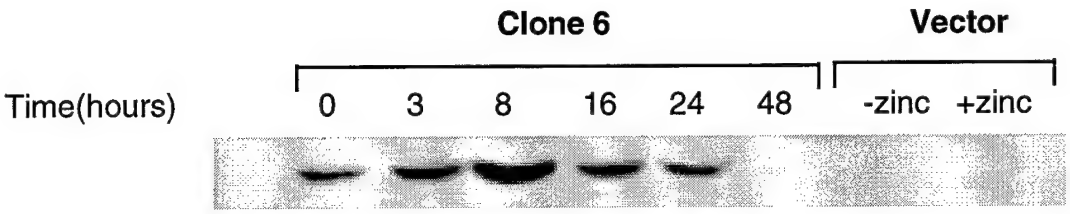


Figure 3. Western blot analysis of inducible RAK expression during a timecourse.

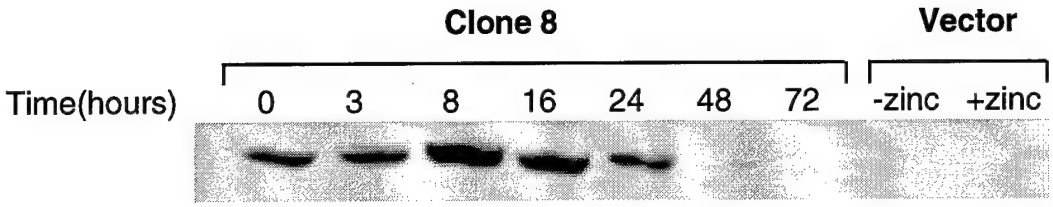


Figure 4.
Production of RAK monoclonal Antibodies

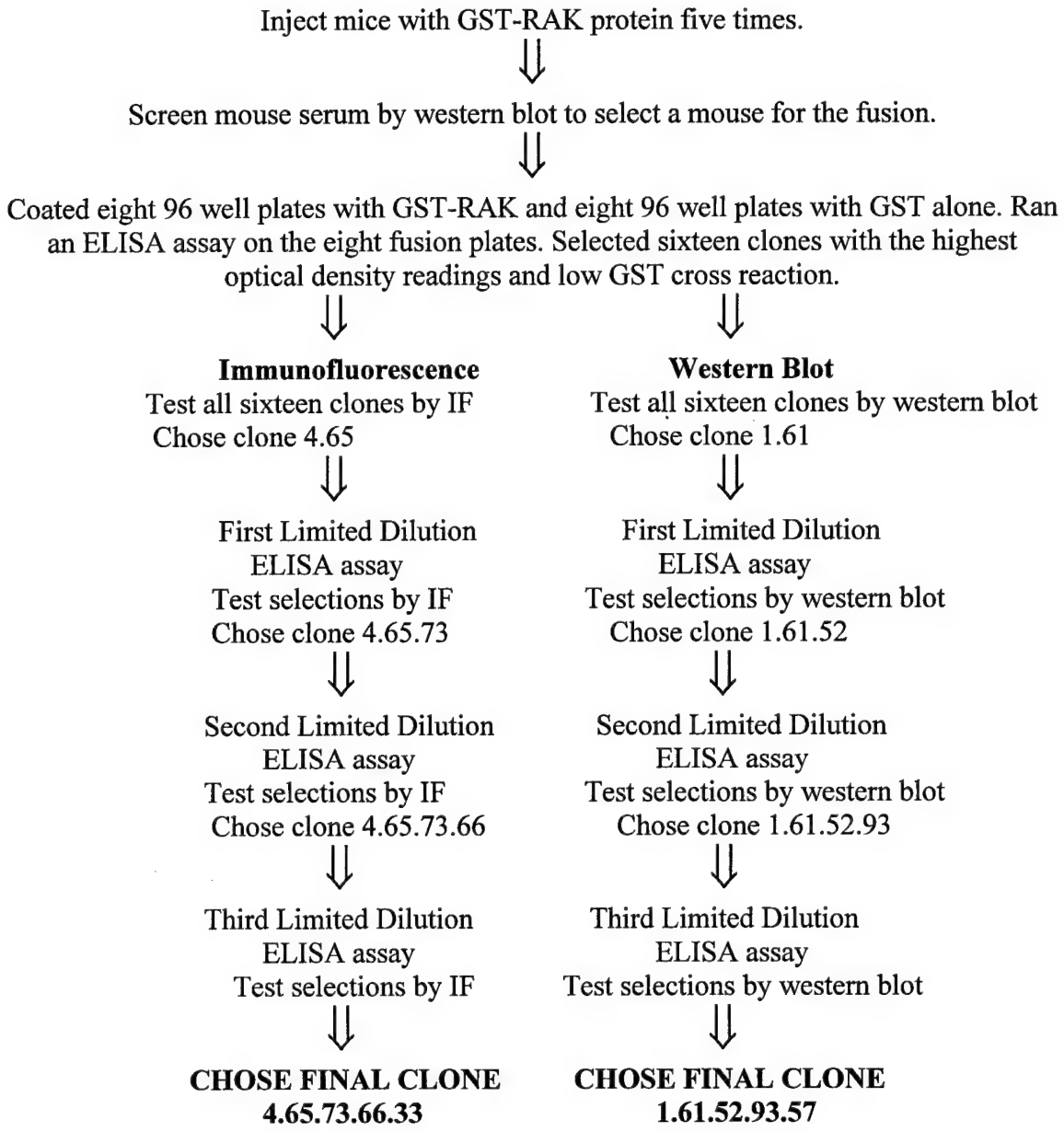


Figure 5. Western blot analysis of the last two limited dilutions of RAK monoclonal antibody 1.61.52.93.17.

An epithelial cell line was transfected with RAK for increased expression, and the antibodies were titered for comparison.

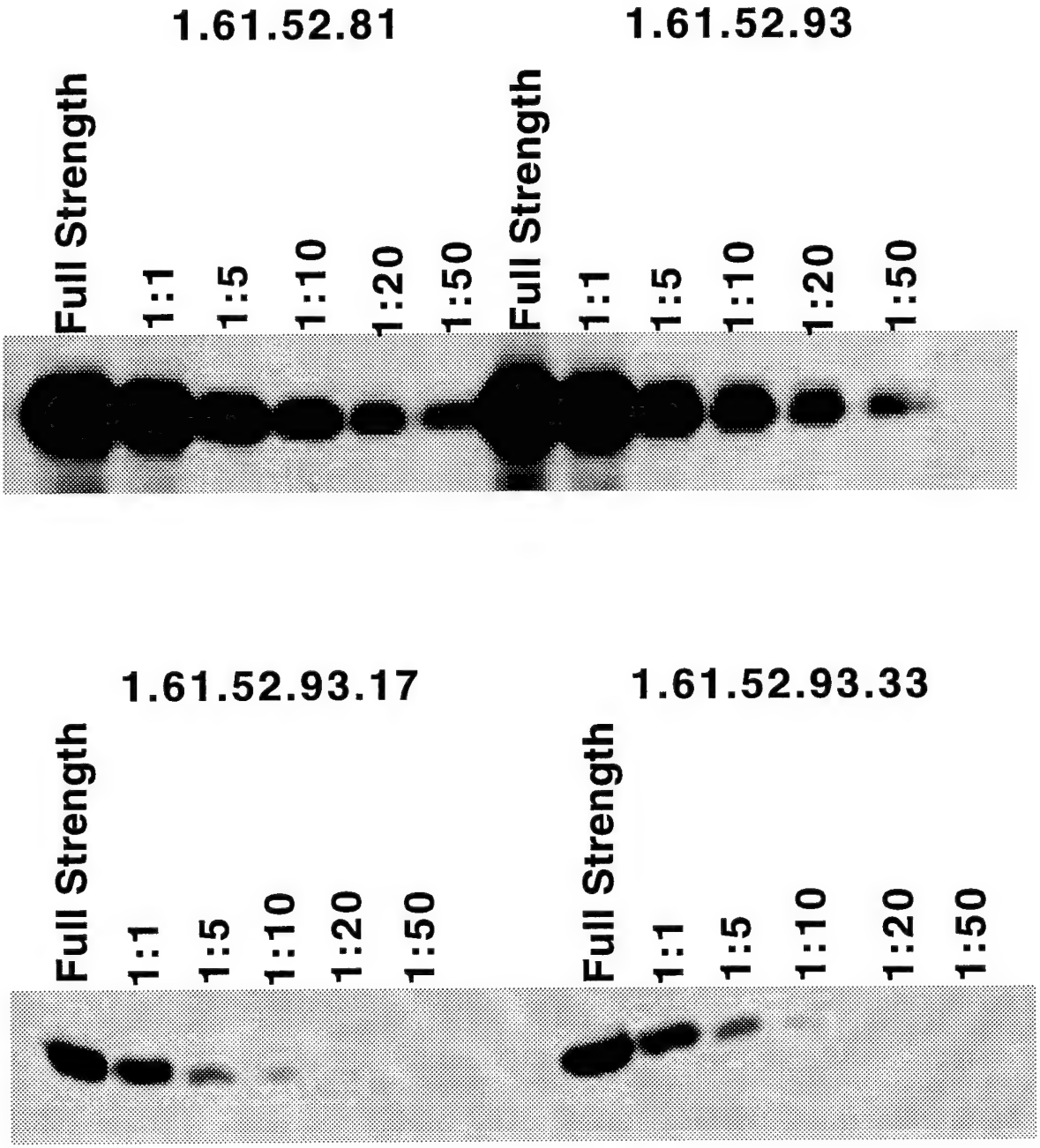
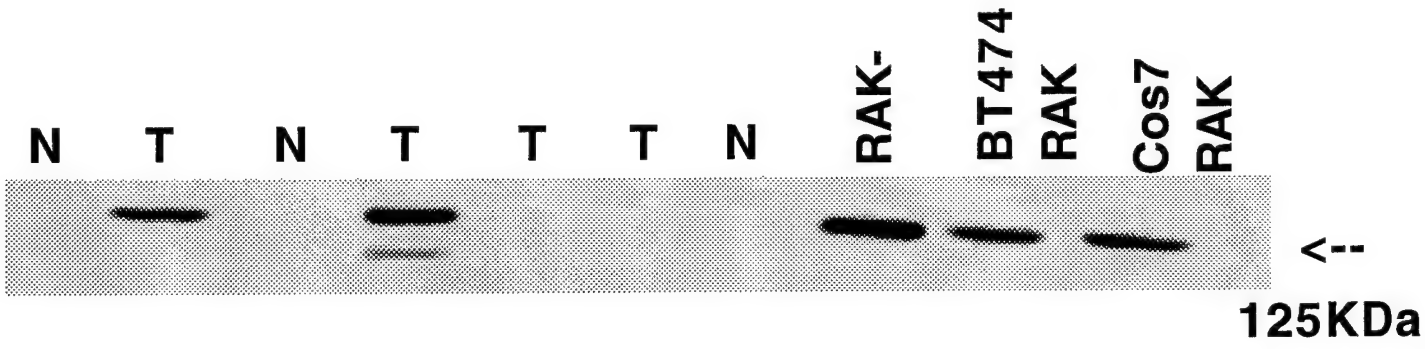


Figure 6. Western blot analysis of RAK expression in human breast tissues using the monoclonal antibody 1.61.52.93.17

A. anti-RAK monoclonal antibody 1.61.52.93.17

B. control anti-FAK monoclonal antibody 3.95



N=normal tissue T=tumor RAK- = RD, a mesenchymal cell line that does not express RAK

The lower panel displays staining for Focal Adhesion Kinase, which we have shown is upregulated in tumors

III. Conclusions

These results show that Rak is a potent growth inhibitory tyrosine kinase expressed in human breast cancer. We conclude that breast cancer cells are exquisitely sensitive to manipulations in their endogenous levels of Rak expression, as induction of this kinase in breast cancer cells led to apoptosis. We also conclude that Rak is expressed in normal breast tissue and appears to be expressed in a subset of human breast cancers. Further work is necessary to characterize the nature of the gene product expressed in breast cancers, and to determine the mechanism of growth inhibition in the breast cancer cells.

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Appendix A

Individual Methods

A. ELISA Assay as a Monoclonal Antibody Screen

The purpose of the ELISA screen is to test whether the limited dilutions of the antibodies in question are recognizing the initial immunogen and which of these dilutions are the most reactive. Briefly, the solid matrix is coated with the original immunogen, gstRakNT, at a concentration of 7.5μg/ml. Excess protein is washed from the plate and the serum is allowed to bind to the immunogen, After washing away the unbound antibodies, the specifically-bound antibodies are detected with an HRP-conjugated secondary antibody recognizing the mouse Ig using the reagent pNPP (Sigma). Signal intensities are read at 405 nm wavelength. Clones that offer the most intense signals are expanded slightly for closer analysis such as by Western, immunoprecipitation, or immunofluorescence.

B. Western Analysis

Western analysis methodologies are used to select monoclonals which can detect Rak on the solid support (nitrocellulose, Immobilon, or nylon filter). We transfer 250 μg of RakNT Cos7 lysate from an SDS-PAGE gel to Immobilon filter. The filter is mounted to a multichannel rig after blocking in 1x blotto, where many different serum samples can be compared on the same filter and lysate. The unbound antibodies are washed away and bound antibodies are detected with HRP-conjugated goat antimouse antibodies followed by ECL detection reagent (Amersham). It is useful, once a Western-positive antibody has been identified, to dilute the antibody to determine the functional concentration to be used. Once this concentration is determined, it is also useful to attempt to block the reactivity by incubating the diluted antibody with gstRakNT before applying it to the multichamber rig. A blocked Western signal is a good indication that the antibody is indeed specific for Rak.

C. Immunoprecipitation

The purpose of immunoprecipitation is to show the monoclonal binds to native Rak within a cell lysate preparation. In our procedure, we first preclear 500μg Cos7 RakNT lysate with protein A and G/Sepharose. The lysates are spun and supernatant is transferred to a fresh tube. Mouse serum supernatants are added to the lysate supernatants, along with more protein A and G/Sepharose. After this incubation, the bead/mAb/RakNT complexes are pelleted, washed, resuspended in gel loading buffer, and separated by SDS-PAGE. The proteins are transferred to Immobilon where they are visualized by standard Western techniques.

D. Indirect Immunofluorescence Detection

It is extremely useful in our research to have an antibody reagent which specifically sees Rak *in situ* so that we can tell where it is localized in overexpression systems, tissues and paraffin samples. In this procedure, BT474 cells are allowed to attach to coverslips in media containing FBS. The cells are washed, fixed with 3.7% paraformaldehyde for 15

minutes, rewashed, and permeabilized using 0.1% Triton X-100 for 3 minutes on ice. The cells are washed again and blocked with 10% normal goat serum for 30 minutes before Rak antibody is added to the cells. Antibody is incubated with the cells for one full hour and washed away. A rhodamine conjugated secondary antibody is added and incubated for 45 minutes, washed, mounted and viewed using a fluorescent microscope.

REVISED STATEMENT OF WORK FOR 1998 ANNUAL REPORT

Technical Objective 1

a. Isolate and characterize breast cancer cell lines containing a metallothionein promoter: months 18-28.
b. Mutate the kinase domain, and if relevant, the amino-terminal protein interaction domains of Rak and determine their biological activity: months 28-48.

Because this project has undergone a change of Principal Investigator, I would like to formally submit my plans for the future of this Technical Objective, as suggested in the review of our last Annual Report. I feel that the ability of Rak to rapidly inhibit the growth of breast tumor cells warrants further investigation to understand the nature of this effect in breast cancer cells and to develop translational therapeutics based on Rak. My lab has developed a large amount of experience with inducible promoters in breast cancer cells, and are presently submitting a manuscript for publication regarding this work. We are able to introduce a potent growth inhibitory gene, a carboxy terminal variant of the Focal Adhesion Kinase, into breast cancer cells and induce its expression. We then monitor expression by immunofluorescence, and analyze the resulting phenotype by TUNEL assay for apoptotic cells and FACS analysis for any block in the cell cycle.

We have already sub-cloned the full-length Rak cDNA into the pMEP4 plasmid and have introduced this plasmid into BT474 breast cancer cells. This cell line was omitted from our earlier analysis for logistical reasons, but we have since developed a series of techniques with this cell line. We also plan to introduce this plasmid into BT-20, MDA231, and MCF7 cells, depending on our initial experiments with the BT474 cells. The pMEP4 plasmid contains a metallothionein promoter, in which the expression of an inserted gene can be induced by simply adding zinc to the tissue culture medium. This results in minimal toxic side effects, and we have optimized both the selection for cells containing the expression plasmid and the induction conditions.

As implied above, we will assess the induction of Rak gene expression by Western blot analysis with the M2 Flag epitope tag antibody (the Rak cDNA is cloned in frame with the FLAG epitope tag sequence). We will use immunofluorescence with the M2 anti-FLAG antibody to determine the number of induced cells expressing high levels of Rak and to analyze their morphology. Because of our transient transfection experiments, we anticipate that cells will lose adherence following Rak expression and float from the dish. In this case, we will determine whether they have undergone apoptosis by assessing their ability to exclude Trypan Blue, by staining their DNA with Hoechst stain and examining whether they have fragmented nuclei, and by TUNEL assay. If the cells do not appear apoptotic, we will analyze them by FACS analysis to determine whether they have arrested at a particular point in the cell cycle. This is important, because Dr. Liu's lab has already found that expression of Rak in mesenchymal cells and in COS-7 kidney cells causes arrest at the G2/M transition. Thus, G2 arrest is a plausible result for this experiment.

If Rak induces apoptosis or arrest at the G2/M transition, we will return to Technical Objective 1a, which calls for a systematic analysis of the structural features causing this growth arrest. First, we will determine whether growth arrest requires kinase

activity by performing the same experiments with a kinase inactive variant of Rak. If this still is capable of inducing G2 arrest, we will try deletion of each of the SH2 and SH3 domains (the structure of Rak is shown in a cartoon diagram in Figure 1). While a number of other experiments are easily imagined along this line, we will await the next results before a more extensive survey of the possible sites of Rak that can be mutated will be reviewed. Our ultimate goal is to determine the nature of the Rak-mediated growth arrest, and assess whether this can be used as a therapeutic target for breast cancers.

Figure 1. The Rak domain structure

Structural map of the Rak coding sequence, showing the amino-terminal SH2 and SH3 domains. The site for the K262R mutation within the ATP binding pocket of Rak is also shown. This mutation results in a kinase-inactive mutant of Rak.

Technical Objective 2

a. Prepare monoclonal antibodies for analysis of Rak in breast tumors: months 18-24
b. Analyze Rak expression in breast tumors by Western blot: months 24-30
c. Analyze Rak expression and localization in breast tumors by immunohistochemistry: months 24-48.

Our initial characterization of Rak indicated that Rak is expressed in 1/3 of breast cancers at the RNA level. This was a small study, with only 9 samples. Since then, we have used a polyclonal antibody targeted to a GST fusion protein containing the Rak carboxy terminus to study Rak expression in a larger number of samples. At the protein level, we have found that Rak was expressed in 37 out of 100 breast tumors. However, there was a prohibitive amount of background on these blots, making any sort of densitometric quantification difficult.

For this reason, I propose to prepare a monoclonal antibody to Rak. The amino terminus of Rak contains a 57 amino acid unique region which contains only a slight degree of homology to other known proteins. We have prepared a GST fusion protein containing this region for use as an immunogen. This protein has been injected into mice,

and spleen cells from these mice have been fused to create hybridomas. We have screened 768 hybridoma cultures, and have found 1 isolate which recognizes Rak by Western blot and 1 isolate which recognizes Rak by immunofluorescence on BT474 breast cancer cells. Because Rak is expressed in only a limited number of cell lines, we can readily estimate the specificity of this staining by comparing staining for Rak in BT474 breast cancer cells with staining in a mesenchymally-derived cell line such as the RD rhabdomyosarcoma cell line.

If our preliminary results with these antibodies continue to be positive, we will soon have reagents to study a number of characteristics of Rak. I propose the following Objectives:

1. Study the expression of Rak in a large (>100) number of primary breast tumors. We collaborate with Dr. Gary Clark of the University of Texas at San Antonio, and have access to large numbers of breast cancer samples, 200 of which have clinical followup. We will analyze Rak expression in cohorts of these samples and correlate the levels of expression with other phenotypical and biological markers of breast cancer which have been measured in these samples. Thus, we can ask, for example, whether Rak expression correlates with clinical stage, outcome, hormonal receptor status, histological grade, ploidy, and S-phase fraction. These will be the first population-based studies of Rak expression and will be performed with appropriated biostatistical support from UNC Lineberger Comprehensive Cancer Center.

2 We have previously found that Rak localizes to the nucleus in a subset of breast tumors and cell lines. I propose to stain tumors which are positive for Rak expression by Western blot by immunohistochemistry and determine whether Rak is localized to the nucleus or the cytoplasm in these tumors. These experiments will be done on fresh, OCT-embedded breast cancer specimens obtained through the UNC Lineberger Comprehensive Cancer Center Tumor Bank, which I direct. Through these analyses, we can correlate both the levels of Rak expression and the nuclear vs. cytoplasmic localization of Rak with clinical stage, histological grade, and ploidy and cell cycle status. Pending these results, we will then develop methods of immunohistochemical characterization of Rak on formalin-fixed, paraffin-embedded tissues in order to study large samples for more extensive clinical correlations, as described above.

Technical Objective 3

a. Screen for deletions of the Rak gene in DNA of breast tumors (dependent on the results from Technical Objective 2b): months 30-48

We have previously found that Rak localizes to chromosome region 6q21, a region that is deleted in approximately 1/3 of human breast tumors. Interestingly, when we studied Rak expression in a panel of 50 breast tumors, we found that Rak expression was decreased or lost in about 1/3 of these tumors. This study was performed with Western blots using a polyclonal antibody to Rak. Before these studies, we analyzed Rak expression in breast tumors by RT-PCR, and once again found that Rak was not expressed in 1/3 of these samples. While these studies suggest a link between Rak and a

region that is lost in tumors, they do not assure that Rak is part of the region that is lost in human breast tumors.

To determine whether Rak is part of the region that is lost, I will begin with our analysis of Rak expression in tumors by Western blot. As shown in the previous section, Rak expression is decreased in some of these tumors. We will extract the DNA from all of these tumors and analyze the stability of the Rak gene by Southern blot following restriction enzyme digestion with enzymes that are known to cut Rak, including BamHI, HindIII, and EcoRI. Initially, we will probe the blots with probes derived from the cDNA sequence. If this is problematic, we will probe blots with fragments of the genomic sequence of Rak. Due to the large size of this fragment, we anticipate that the cDNA sequence will be more convenient as a probe.

I had originally proposed to begin these studies at month 30 of this grant, and at present we are on schedule. DNA is presently being prepared from a panel of breast tumors, and we will begin Southern blot analysis shortly.